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Full Length Research Paper

Investigation of genetic variability related to the *in vitro* floral hermaphroditism induction in Date palm (*Phoenix dactylifera* L.)

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This paper reports on a molecular analysis study conducted on Date palm flowers from the Deglet Nour cultivar to investigate putative genetic variability related to the *in vitro* floral hermaphroditism induction. Natural male and female as well as hermaphrodite ones that were produced *in vitro* through the hormonal treatment of female flowers were submitted to ISSR-PCR analysis. Microsatellite based amplification (ISSR) was applied on genomic DNA from inflorescences taken at different periods of hormonal treatment corresponding to the various deviation stages to search for putative variations that may have occurred on the initial genome due to the application of plant growth regulators. Several amplification bands were purified, cloned, and sequenced. The results revealed that hormonal treatment entailed no detectable genetic variation in the treated Date palm flowers. Two of the selected and ISSR-PCR amplified DNA fragments showed however, possible links with flowering regulation. The findings indicate that these sequences are potential candidate gene markers that may enhance our understanding of flower development and sex identification in this species.

Key words: Date palm, female inflorescences, hermaphrodite flowers, *in vitro* culture, ISSR, sex identification.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a monocotyledonous and dioecious species grown particularly in the arid and semi-arid areas of the world. It has immense socio-economic, environmental and ecological values in several North African and Middle-Eastern countries. The expansion of its cultivation is, however, often hampered by several constraints particularly those pertaining to the relatively slow sex identification methods. In fact, Date palm reproduction leads to a very heterogeneous population composed of male and female plants in almost

equal proportions (50% each). The latter remain indistinguishable until their flowering phase. Since fruits are borne only by female Date palm trees and since this species is characterised by a delay in flowering to reach the reproductive stage (6 to 8 years), the search for effective methods of early sex identification requires a genetic approach.

Date palm inflorescence is composed of an axis that divides into branches (pedicels) carrying the flowers. The latter are unisexual and develop on distinct plants. Each inflorescence is enveloped with a big bract called spathe whose shape constitutes a sex characteristic. The inflorescences are derived from the development of the inflorescences buds located at the leaf armpits (Masmoudi-Allouche et al., 2009).

Date palm flowers are trimeric and unisexual. In addition to the three sepals and three petals of the flowers, the male flower is characterized by the development of an androecium composed of six stamens

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Abbreviations: IBA, Indole-3-butyric acid; BAP, 6-benzylaminopurine; PCR, polymerase chain reaction; ISSR, inter simple sequence repeat; DNA, desoxyribonucleic acid.

formed in two whorls of three stamens each. The male flower also contains three vestigial carpels. The female flower, however, develops a gynoecium composed of three free carpels, and it also contains six vestigial stamens (staminodes) (Masmoudi-Allouche et al., 2009).

At present, various effective strategies are employed to control Date palm micropropagation (Drira and Benbadis, 1985; Fki et al., 2003). Its dioecy, its recalcitrance, and unknown genetics remain; the major constraints that hinder the efficient application of biotechnologically-based Date palm selection programs. A previous study by the authors attempted to help overcome some of the difficulties that relate to the characteristics of the Date palm (Masmoudi-Allouche et al., 2010). In fact, an *in vitro* flowering process was induced for several varieties and the sex type of young plants was successfully determined within only few months.

The authors have also reported on the successful induction of hermaphrodite flowers on female ones (Masmoudi-Allouche et al., 2009). In fact, this female-to-hermaphrodite flower sex modification was achieved for several Date palm cultivars under particular tissue culture conditions using an appropriate hormonal treatment based on the addition of IBA (indole-3-butyric acid) and BAP (6-benzylaminopurine) at different concentrations. In this context, particularly the generation of genetic variation through the *in vitro* application of plant growth regulators, the effect of such hormonal treatment on genetic traits of female flower was achieved for the elite Deglet Nour variety from the initial (female) to the final (hermaphrodite) stages.

This study aimed to use Inter Simple Sequence Repeat (ISSR) analysis to search for genetic markers related to the floral hermaphroditism induction and/or involved in the sex determination of Date palm. In fact, the data currently available on genetic sex determination is very scarce and, to the authors' knowledge, only one report, by Siljak-Yakovlev et al. (1996), showed that Date palm sex determination is of the XY type where male plants have heteromorphic chromosomes and female plants have homomorphic ones.

A number of molecular techniques have been used to identify molecular markers related to both cultivar identification and sex determination in Date palm. In this context, RAPD markers were employed by Corniquel and Mercier (1994) for Date palm cultivar identification. This technique was also used by Bekheet et al. (2008) for early identification of sex type in Date palm. Such markers, however, lack reproducibility. Accordingly, this study opted for the application of an ISSR-PCR technique. The latter has frequently been reported to generate more reproducible data. This is in part due to the fact that microsatellites are abundant in the eukaryotic genome and polymorphic in length, which leads to the generation of several fragments by amplification (Tautz, 1989; Gupta et al., 1994; Zietkiewicz et al., 1994; Zahdi et al., 2002; Zehdi et al., 2004). This technique was, in fact, previously

applied in several molecular phylogenetic studies on Tunisian Date palm cultivars by Zahdi et al. (2002, 2004). It has also been employed for the identification of sex marker in a variety of other plant species, such as *Carica papaya*, for which the (GATA)_n microsatellite sequence was identified as a sex marker (Parasnis et al., 1999). Accordingly, this study was undertaken with the aim of investigating any putative genetic variation that may affect the female inflorescence genome during hormonal treatment.

MATERIALS AND METHODS

The experimental assays of this study were performed on natural female Date palm flowers from Deglet Nour variety and on hermaphrodite flowers that were formed from female flowers cultured *in vitro* and subjected to appropriate hormonal treatment as previously described by Masmoudi-Allouche et al. (2009). The female inflorescences were collected from Date palm plants growing in the south of Tunisia and sterilized with 0.01% HgCl₂ solution. They were then rinsed with sterile distilled water and cultured on a MS (Murashige and Skoog, 1962) basal medium supplemented with a combination of IBA and BAP growth hormones. These plant growth factors were added at concentrations of 4.92 and 4.44 μM, respectively. After about 15 days of culture in darkness at 27°C, *in vitro* proliferation of stamens within the female flowers was induced, leading to the production of hermaphrodite flowers that are both staminate and pistillate (Masmoudi-Allouche et al., 2009). The female inflorescences used were taken at several culture periods during their reversion to hermaphrodite state after different day treatments.

Date palm male flowers (m₁, m₂, m₃ and m₄) collected from different male plants cultivated under field conditions in Tunisia were used for comparison.

Total DNA extraction

Total DNA (desoxyribonucleic acid) was extracted from female flowers at different deviation stages using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The protocol was as follows: - approximately 40 mg of flowers were grinded in liquid nitrogen, 600 μl of Nuclei Lysis solution was added and incubated at 65°C for 15 min. 3 μl of RNase solution was added and incubated at 37°C for 15 min and the sample was cooled to room temperature for 5 min. Protein precipitation solution was added and was incubated for 5 min on ice. Centrifugation was done at 16,000 g for 3 min. The supernatant was transferred to a clean tube containing room temperature isopropanol and mixing was done by inversion and centrifugation at 16,000 g for 1 min. The supernatant was decanted, room temperature 70% ethanol was added and was centrifuged at 16,000 g. Aspiration of the ethanol was done and the pellet was air-dried. DNA rehydration solution was added and rehydration was done at 65°C for 1 h or overnight at 4°C.

Total DNA was extracted from male flowers as described by Dellaporta et al. (1983). The stock of DNA samples was diluted in sterile distilled water to make a working concentration of 10 ng. μl⁻¹ used in PCR analysis.

DNA analysis by ISSR –PCR technique

The primers

This study used eight microsatellite ISSR primers that had the

Table 1. Primers used for the ISSR-PCR amplification.

Primer name	Primer sequence	Annealing temperature (°C)
PW2	(AG) ₁₀ C	60
PW3	(AG) ₁₀ T	57
PW4	(CT) ₁₀ A	57
PW5	(CT) ₁₀ G	60
PW6	(CT) ₁₀ T	57
PW7	(CAG) ₅	52
P016	(CA) ₆ CC	46
P06	(CA) ₆ GT	46

following sequences: PW2: (AG)₁₀C with an annealing temperature (T_m) of 60°C, PW3: (AG)₁₀T with a T_m of 57°C, PW4: (CT)₁₀A with a T_m of 57°C, Pw5: (CT)₁₀G with a T_m of 60°C, Pw6: (CT)₁₀T with a T_m of 57°C, PW7: (CAG)₅ with a T_m of 52°C, P016: (CA)₆CC with a T_m of 46°C and P06: (CA)₆GT with a T_m of 46°C (Table 1).

Most of these primers were previously described in earlier studies involving the polymorphism analysis of different Date palm varieties (Zehdi et al., 2004). The amplification reactions were performed in a 25 µl reaction mixture containing 50 ng DNA, 1 U Taq DNA polymerase (Bioline), and 10X reaction buffer including 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 200 ng of the primer. The amplifications were carried out on total DNA using a 2700 thermo cycler (Applied Biosystems) set as the following parameters: 5 min at 94°C; 35 cycles (30 s at 94°C, 90 s at the annealing temperature of the primer used, 90 s at 72°C) and 5 min at 72°C.

Analysis of the amplification products

The amplification products were analysed in 6% polyacrylamide gel electrophoresis using TBE 1X buffer (0.089 M Tris HCl, 0.089 M Borate, 0.2 mM EDTA, and pH 8.3) (Sambrook et al., 1989). DNA bands were visualized by ethidium bromide (1 µg.ml⁻¹) staining.

The eluted fragments were inserted in a pCR[®]2.1 vector (Invitrogen) using the TA Kit cloning according to the manufacturer's instructions. The ligation mixture was transferred to *Escherichia coli* TOP10 strain. The bacteria were cultured in an LB medium (10 g l⁻¹ Tryptone; 5 g l⁻¹ yeast extract, 5 g l⁻¹ Na Cl, 16 g l⁻¹ agar, and pH 7 to 7.4) supplemented with 100 µg ml⁻¹ ampicillin. Competent cells were prepared using the CaCl₂ method described by Mandel and Higa (1992).

Plasmid DNA extraction

Recombinant DNA plasmids were extracted according to the method of Ish-Horowich and Burke (Ish-Horowich and Burke, 1981). They were digested by *EcoRI*, *BamHI*, and *EcoRV* restriction enzymes and analysed in 1% agarose gel electrophoresis.

Sequencing

A number of DNA fragments were sequenced and compared to those at the NCBI sequence database. The nucleotide sequences of the cloned fragments were determined on both strands using the dideoxy haintermination method (Sanger et al., 1977). Reactions were performed with a thermo sequenase cycle sequencing kit (Amersham) and specific primers. Homology search was performed

using Blast Search algorithm (Altschul et al., 1997).

RESULTS

ISSR-PCR analysis was applied on DNA extract from female flowers subjected to hormonal treatment with IBA and BAP growth hormones at concentrations of 4.92 and 4.44 µM, respectively, which were added to the MS basal medium at the onset of culture. This ISSR analysis was used to detect any potential genetic variability that might have occurred during the culture process. A comparison between male (Figure 1a) and female (Figure 1b) amplification patterns was also performed to identify genetic markers related to sex determination in Date palm. DNA templates were purified from inflorescences taken at different steps of hormonal treatment till the production of hermaphrodite flowers (Figure 1c).

Polymorphic ISSR primers were chosen according to well established data on Date palm genome analysis (Zahdi et al., 2002, 2004). The six PW primers (PW2, PW3, PW4, PW5, PW6 and PW7) did not reveal any polymorphism associated to the hermaphroditism induction process. In fact, none of these primers exhibited variation in the amplification profile of female flowers taken at different hormonal treatment times (Figure 2). Figure 2 illustrates the profiles obtained when PW4, PW5, PW6 and PW7 primers were used. The amplification profiles obtained with those primers also revealed similar profiles between the male and female flowers.

The P06 and P016 primers showed that there was no significant difference in the amplification products from the treated and initial female flowers. Almost the same amplification profiles were obtained for the treated and initial female flower extracts. These findings suggest that this hormonal treatment did not bring about a genetic variability in Date palm flowers.

However, the amplification products obtained with those primers (P06 and P016) revealed a number of differences between male and female flowers (Figure 3). In fact, while the major bands amplified with P06 primer

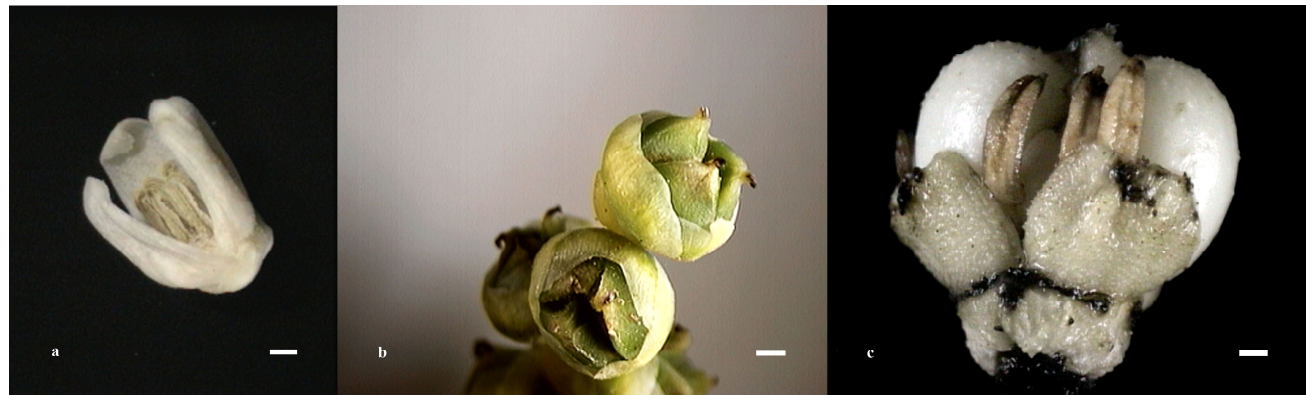


Figure 1. Wild type (a, b) and hermaphrodite date palm flowers differentiated *in vitro* (c). a, Wild male date palm flower; b wild female date palm flowers; c, hermaphrodite flower differentiated under *in vitro* conditions. Scale bars: 1000 μm (a, b), and 600 μm (c).

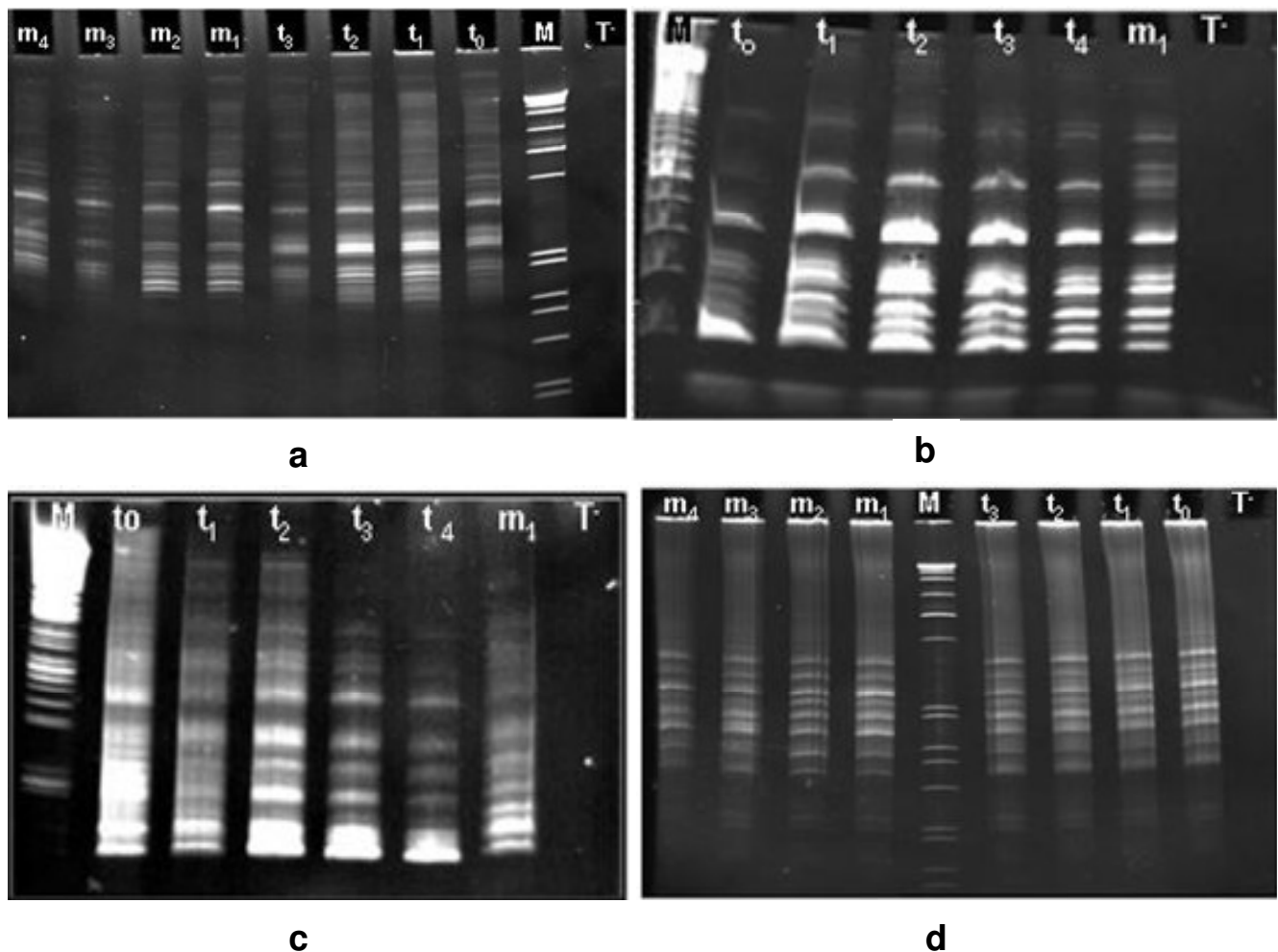
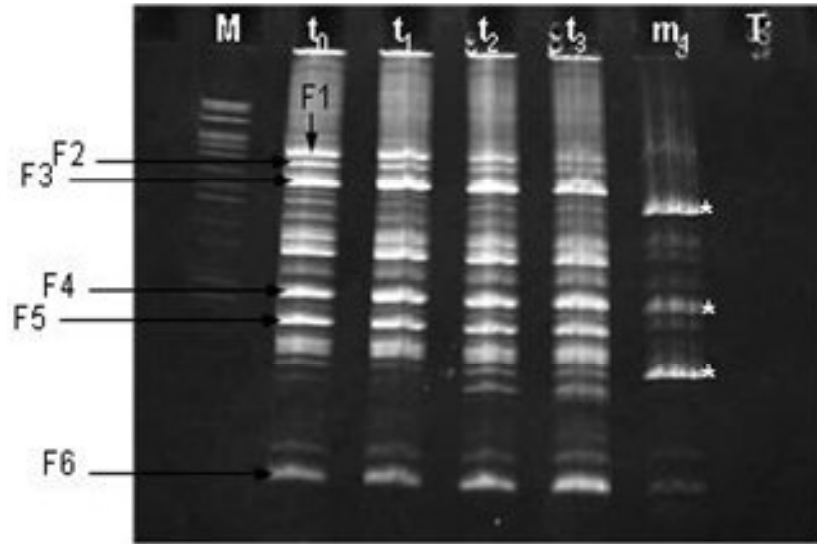
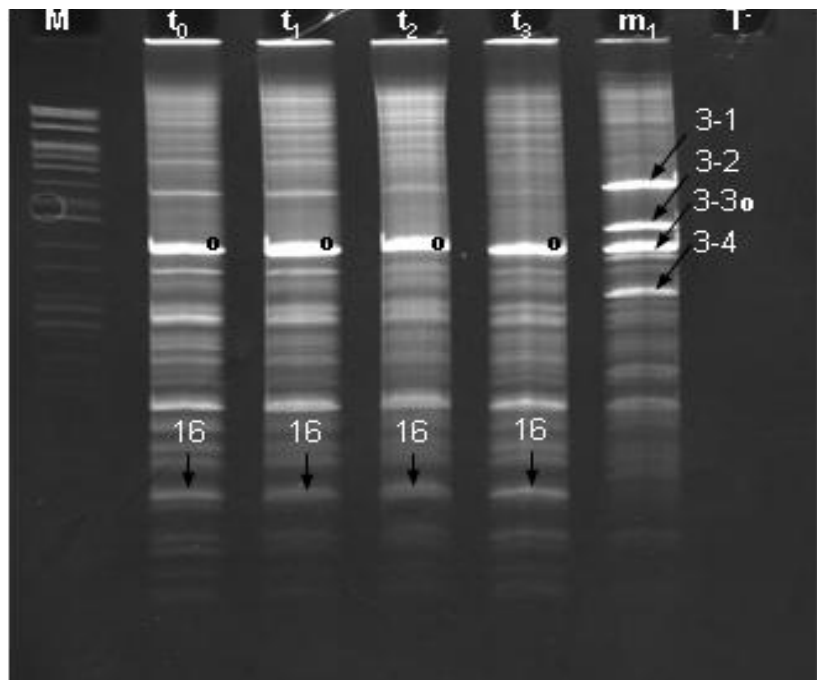


Figure 2. ISSR-PCR amplification products of the different DNA samples in 6% polyacrylamide gel electrophoresis using PW4 (a), PW5 (b), PW6 (c) and PW7 (d) primers. t_0 , Untreated female inflorescences; t_1 , t_2 , t_3 and t_4 , female inflorescences during reversion to hermaphrodite state after 11, 22, 27 and 30 day treatments, respectively; m_1 , m_2 , m_3 and m_4 , male flowers; T, negative control with the reaction mixture excluding DNA; M (PW5 and PW6), *Hinc* II – digested λ DNA (4,78 ; 4,58; 3,51; 3,27 ; 2,84; 2,19; 1,96; 1,79; 1,67; 1,34; 1,26; 1,1; 0,86; 0,73; 0,56 kbp); M (PW4 and PW7), molecular weight marker 1 kbp DNA Ladder (Promega): 12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506; 0.396; 0.344; 0.298; 0.220 ; 0.201; 0.154 ; 0.134 ; 0.075 kbp.



a



b

Figure 3. ISSR-PCR amplification products from the different DNA samples on 6% polyacrylamide gel electrophoresis using P06 (a) and P016 (b) primers. t_0 , untreated female inflorescences; t_1 , t_2 and t_3 , female inflorescences during reversion to hermaphrodite state after 11, 22, and 27 day treatments, respectively; m_1 , male flowers; T, negative control with the reaction mixture excluding DNA; M, *HincII*-digested λ DNA (4.78; 4.58; 3.51; 3.27; 2.84; 2.19; 1.96; 1.79; 1.67; 1.61; 1.34; 1.26; 1.1; 0.86; 0.73; 0.56 kb). *Major bands in male flower profile; O, a major common band between male and female patterns; \rightarrow : Fragments recovered and eluted from the gel.

in the male extract were also present in the female flower profiles, some amplification bands were present only in the female flower extracts (F₁, F₂, F₃, F₅ and F₆). Some of

these DNA fragments were eluted from the gel and inserted into the pCR[®]2.1 vector for sequencing. Amplification with the P016 primer, on the other hand,

Table 2. Sequence comparisons between the sequenced fragments and those available at the data base.

Fragment	Size (kpb)	Origin	Homology	E value	Max identity (%)
F3	1.6	Female	- Cytosolic acetyl- CoA carboxylase (<i>Triticum urartu</i>)	2e-64	73
			-Phosphatidyl serine decarboxylase (<i>Triticum monococcum</i>): ZCCT2, ZCCT1 and SNF2P genes	8e-63	76
			- Cytosolic acetyl- CoA carboxylase (<i>Triticum aestivum</i>)	4e-60	75
F4	0.5	Female	- Peptide transporter (<i>Hakea actites</i>): (PTR4) mRNA	3e-67	79
			- Peptide transporter (<i>Ricinus communis</i>)	1e-60	77
			- Peptide transporter (<i>Oryza sativa</i>): OsTR4	3e-60	77
			- Oligopeptide transporter (<i>Lycopersicon esculentum</i>): (LeOPT1) mRNA	2e-56	76
			- Peptide transporter 1 (<i>Vicia faba</i>): (PTR1) mRNA	3e-55	76
			- Amino acid/Peptide transporter (<i>Prunus dulcis</i>): (PTR2) mRNA	3e-55	76
- Proton-dependent Oligopeptide transporter (<i>Arabidopsis thaliana</i>)	2e-32	74			

revealed three fragments that appeared to be specific only to the male extract (3-1, 3-2 and 3-4) (Figure 3) and a fragment (band 16) that was present only in the female flower extracts (Figure 3). There was also one fragment amplified in both male and female extracts (3-3 in male) (Figure 3).

All of the aforementioned fragments were eluted from the gel and inserted into the pCR[®]2.1 vector for sequencing. The findings revealed that only the F3 and F4 fragment sequences showed homology to known sequences at the NCBI database (Accession numbers: GenBank: EV253702.1 and GenBank: EV253701.1) (Table 2). The other fragments did not match with known sequences.

The F3 DNA sequence showed similarities with phosphatidyl serine decarboxylase mitochondrial gene. This DNA sequence also showed similarities with the cytosolic acetyl coA carboxylase gene. The F4 DNA fragment showed homology with peptide transporter (PTR) gene from several species.

DISCUSSION

This study was aimed to use an ISSR-PCR approach to investigate potential genetic variability related to the *in vitro* floral hermaphroditism induction in Date palm. It investigated the effect of a hormonal treatment (Masmoudi-Allouche et al., 2009) on the molecular state of deviated female flowers during their reversion to a

hermaphrodite state seeking for possible genetic variability associated with hormonal treatment and for potential ISSR sex-specific markers in Date palm. Accordingly, inflorescences were taken at regular times of hormonal treatment and the hermaphrodite ones were subjected to regular analysis. The amplification profiles of male and female flowers were also compared to visualize and search for genetic markers related to sex determination in Date palm.

In fact, the ISSR-marker approach was successfully applied in a previous study on *Carica papaya* where it was reported to have led to the identification of (GATA)_n as a sex related marker (Parasnis et al., 1999). Several other studies have, on the other hand, reported on the convenience of the RAPD method for sex determination (Bekheet et al., 2008). The latter was also applied for other species such *Myristica fragrans* Houtt. (Shibu et al., 2000), *Pistacia vera* L. (Yakubov et al., 2005), and *C. papaya* L. (Parasnis et al., 2000; Urasaki et al., 2002; Chaves-Bedoya and Nuñez, 2007).

The findings revealed that all the ISSR markers used in this study exhibited no differences between the various states of the flowers from the female (*t*₀) to the hermaphrodite state. The observed absence of polymorphism related to the hormonal treatment suggests that the *in vitro* sex change in Date palm flower may be due to a difference in the levels of gene expression. Accordingly, the induction of the male organs (stamens) in the female flowers can be attributed to a disturbance in gene expression regulation caused by hormonal treatment

which could have led to an unusual activation of inhibited male-related loci in female flowers. These results are in agreement with those previously reported by Lebel-Hardenak and Grant (1997) who postulated that in many dioecious species, unisexual floral meristems are sexually bipotent and that a change in the level or ratio of endogenous hormones can trigger a switch between alternative developmental programs of sex-determining genes.

Furthermore, a polymorphism was observed between the male and female flower profiles when ISSR primers P06 and P016 were used. Some of the polymorphic bands were isolated and sequenced. Two of these bands, namely F3 and F4, which were taken from female flowers, showed sequence homology with genes involved in flower and seed developments. The F3 DNA sequence showed similarities with phosphatidyl serine decarboxylase mitochondrial gene. This latter gene seems to be involved in stress response. In fact, it is involved in the modification of the cell membrane lipid composition (phospholipid synthesis) during stress response and also in the synthesis of osmoprotectant amino acids (choline, glycinebetaine) (Larsson et al., 2006).

The F3 DNA sequence also showed similarities with the cytosolic acetyl coA carboxylase gene. The acetyl coA carboxylase (ACC) isoenzymes are responsible for fatty acids (FA) biosynthesis in plastids and mitochondria. The FAs are essential in signalling and facilitating pollen-stigma interactions (Zuther et al., 2004). Zuther et al., (2004) isolated and characterized the promoter of wheat ACC-2,1 gene. They produced transgenic wheat plants expressing a GUS activity under the control of the ACC-2,1 promoter and showed that the expression of this promoter changed during flower and seed developments. Their work demonstrated that the ACC-2,1 promoter is active in the ovary 4 days preceding pollination and that its expression increased to reached a maximum at pollination and then decreased as the seed developed. The authors added that the strong expression of the ACC promoter was confined to the stigma surface of the ovary. The expression in the unpollinated ovary, on the other hand, remained high for several days. Zuther et al. (2004) also demonstrated that no expression was observed in anthers, pollen, or embryos. Overall, the ACC-2,1 gene could be considered a good candidate as a flower maturation marker since its expression seems to be closely related to ovary development and is entirely absent in pollen, anthers, and embryos.

The F4 DNA fragment showed homology with Peptide transporter (PTR) gene from many species. This family of genes is constitutively expressed in all plant tissues (Song et al., 1997). They are transporters involved in the nitrogen (N) uptake from soil and its subsequent transport in planta to support development, growth, and reproduction (Komarova et al., 2008). The PTR genes belong to a large family in planta but have few affiliates in prokaryotes, fungi and animals (Stacey et al., 2002). The

PTR family has only few members in *Arabidopsis thaliana*, 51 PTR genes, which were identified and divided into four subfamilies (Stacey et al., 2002). The AtPTR2 seems to be involved in flowering and seed development. The AtPTR5 is expressed in pollen, ovules and early seed development (Komarova et al., 2008). The constitutive expression of the antisense sequence of the AtPTR2 gene in transgenic *A. thaliana* plants has led to a flowering delay and an arrest of seed's development (Song et al., 1997). This F4 DNA fragment, which showed homology with a peptide transporter that was apparently involved in the flowering and seed development processes, is common to both male and female flowers and can be used as a candidate gene for flowering regulation.

In fact, the study of the expression of the aforementioned genes during male, female, and hermaphrodite flower developments may facilitate the understanding of the mechanisms involved in Date palm flowering and regulation. The cloning of the full length genes corresponding to F3 and F4 fragments and the study of their expression profiles during flower development in Date palm can provide useful information about the regulation of this process.

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